AMENDMENTS TO THE SPECIFICATION:

Please delete the paragraphs on page 3, lines 3-26 and replace them with the following paragraphs:

T lymphocytes obtained from tumor infiltrated lymph nodes were repeatedly stimulated in vitro with the autologous tumor and T cell clones were generated by limiting dilution. CD4+ T cells clones recognizing the autologous tumor in an HLA-DR restricted fashion were obtained, characterized in vitro for their fine specificity and used as cellular probe in a genetic approach aimed at defining the molecular nature of the recognized antigen. The screening of a cDNA expression library constructed using as template the RNA of the autologous melanoma led to the identification of the tyrosine phosphates receptor K gene (R-PTP-K) as encoding the antigen recognized by the CD4+ melanoma specific clones. The R-PTP-K mRNA cloned by melanoma cells contains a non-conservative Gly→Arg mutation in the fourth fibronectin III-like domain of the protein. This amino acid change generates a T cells epitope presented by the HLA-DRβ1*1001 that is recognized by the CD4 T cell clone used to screen the tumor cDNA library and by all the 5 different clones isolated from the tumor infiltrated lymph nodes of the same patient. The antigenic epitope was identified in the region 667-682 of $PTPRK_{Gly677\rightarrow Arg682}$ and it has sequence PYYFAAELPPRNLPEP (SEQ ID NO: ~~N.~~ 1).

A first aspect of the invention is directed to the immungenic peptide of SEQ ID NO: ~~N.~~ 1 and the use thereof in the generation of antibodies and/or T helper or cytotoxic cells, more generally in the induction of a tumor-specific immune response, for diagnostic or therapeutical applications, in particular for the diagnosis, prevention or immune therapy of tumors expressing $PTPRK_{Gly677\rightarrow Arg682}$.

Please delete the paragraphs on page 4, line 25 to page 6, line 11 and replace them with the following paragraphs:

In a further embodiment, the invention provides polyclonal or monoclonal antibodies, fragments or derivatives thereof such as Fab, Fv or scFv, able to recognize and bind the peptide SEQ ID NO: ~~N.~~ 1. The isolated antibodies can be used in tumor immune therapy or in immune diagnostic techniques for the definition of tumors expressing $PTPRK_{Gly677 \rightarrow Arg682}$.

In a yet further embodiment, the invention provides isolated CD4+ T cells specifically recognizing a tumor expressing $PTPRK_{Gly677 \rightarrow Arg682}$ and the use thereof for inducing a cell-mediate immune response against such tumor. These cells can be isolated from PBMC obtained from the patient to be subjected to the treatment, and they can be activated in vitro with the peptide SEQ ID NO: ~~N.~~ 1, optionally in the presence of cytokines, or using cells carrying the peptide in association with HLA-Class II molecules, such as APC (antigen presenting cells) expressing the allele HLA-DR β1*1001 loaded with the peptide. APCs can be genetically modified, e.g. by transfection with a viral or retroviral vector, so as to express the specific allele HLA or the peptide or a precursor thereof. Modified HLA cells can be used to activate T cells either in vitro or in vivo. In vitro activated T cells can be subsequently reintroduced in the patient to prevent the onset, to arrest the growth or to reduce the amount of tumor cells. Before being reintroduced into the patient, lymphocytes may be purified, for example by means of an affinity column using an antibody directed against CD4 or other markers.

In a further embodiment the invention provides an isolated nucleic acid molecule encoding the epitope of $PTPRK_{Gly677 \rightarrow Arg682}$ herein described, preferably the sequence CCGTATTACTTTGCTGCAGAACTCCCCCCGAGAAACCTACCTGAGCCT (SEQ ID NO: ~~N.~~ 2)
as well as a vector and a host cell including said sequence. DNA molecules containing the peptide-encoding sequence, or a part thereof, and the gene constructs thereof can be used in the vaccination of subjects at risk of developing tumors, particularly melanoma, or cancer patients. DNA immunization can be carried out according to known techniques (Donnelly J.J. et al., 1994, The Immunologist 2:1). The intramuscular administration route is preferred, but also the parenteral and mucosal routes can be used (pnas 1986, 83, 9551; wo90/11092). Moreover, DNA can be adsorbed onto gold particles for the subcutaneous administration with a biolistic apparatus (Johnston, 1992 Nature, 356, 152).

In addition to the above described therapeutic uses, nucleic acid molecules containing the peptide-encoding sequence, or a part thereof, as well as the peptide itself, can be used in the diagnosis of melanoma expressing $PTPRK_{Gly677 \rightarrow Arg682}$, for instance by PCR analysis or immunoassays using epitope-specific antibodies. Furthermore, complexes between the peptide SEQ ID NO: ~~N.~~ 1 and HLA-DR β1*1001 cells can be used for monitoring in vitro or ex vivo the immune response of subjects vaccinated with the peptide.

Please delete the paragraphs on page 8, line 3 to page 10, line 5 and replace them with the following paragraphs:

Characterization of cDNA #11. cDNA #11 and related minigenes are represented as boxes aligned to a schematic structure of PTPRK protein. Black square in each minigene

indicates the position of an ATG codon in frame with the starting ATG of the full-length gene (GenBank NM_002844). The mutated nucleotide (g→a) occurring at position 2249 is indicated. Minigenes were synthesized by PCR amplification of cDNA #11 using an identical forward primer (F2) coupled with different, nested reverse primers mapping downstream the mutation (EPR1, EPR2, EPR2WT, and EPR3 reverse primers, indicated by the arrows). Minigenes were cloned into expression vector pcDNA3/TOPO and then co-transfected with pcDNA3-DRB1*1001 or pcDNA3-DRB1*0102 into CIITA$^+$-293 cells. Clone TB515 (1×10^5 cells/well) was added to each transfectant, and after 24 h supernatants were evaluated for the content of IFN-γ by ELISA. In the table: +, positive recognition by TB515; -, no recognition by TB515. EP2wt minigene contained the non-mutated (g) nucleotide. Amino acid sequence in the bottom of the figure was deduced from the sequencing of cDNA #11. Abbreviations in the figure: LS, leader sequence; MAM, meprin/A5/R-PTP□ motif; Ig, immunoglobulin-like domain; FNIII, fibronectin type III-like domain; TM, transmembrane; PTP, protein-tyrosine phosphatase domain; R, arginine deriving from the nucleotide g→a mutation. Figure discloses SEQ ID NO: 21.

Figure 5

Identification of the TB515 epitope. (A, B, C) LCL15392 cells (5.0×10^3 cells/well) pulsed for 2 h at 37°C with the synthetic peptides in different concentrations were used to stimulate TB515 T lymphocytes. The TB515 (5×10^3 cells/well) was added, and after 18 h medium was collected and IFN-γ measured by ELISA. Identical results were obtained using LCL3700, sharing only the DRB*1001 with pt15392 (not shown). (D) 5×10^3 LCL15392 cells were incubated with various concentrations of the competitor peptides for 15 min. Competitor peptides included: PYGFAAELPP**R**NLPEP (SEQ ID NO: 26), modified in

position 3, the wild type PYYFAAELPPGNLPEP (SEQ ID NO: 23), and the HLA-A3 binding peptide ILRGSVAHK (SEQ ID NO: 28) which was used as negative control. PYYFAAELPP**R**NLPEP (SEQ ID NO: 1) peptide was then added at 100 nM. After 1 hr of additional incubation at 37°C, TB515 (5×10^3 cells/well) was added, and after 18 h medium was collected and IFN-γ measured by ELISA. Identical results were obtained using LCL3700 sharing only the DRB*1001 with pt15392. Mutated amino acid is written in bold; substituted amino acids are underlined. Peptides disclosed as SEQ ID NOS: 1 and 22-28, respectively, in order of appearance.

Figure 6

Peptide specificity of T lymphocyte clone TB48. LCL15392 cells (5.0×10^3 cells/well) pulsed for 2 h at 37°C with the synthetic peptides in different concentrations were used to stimulate TB48. The TB48 clone (5×10^3 cells/well) was added, and after 18 h medium was collected and IFN-γ measured by ELISA. Identical results were obtained using LCL3700, sharing only the DRB*1001 with pt15392. Peptides disclosed as SEQ ID NOS: 1, 23 and 26, respectively, in order of appearance.

Figure 7

PTPRK epitope specific immunity in PBMCs of pt 15392. PBMCs of pt15392, obtained during the disease-free period 12 months after surgery, were stimulated in vitro with the mutated PYYFAAELPP**R**NLPEP (SEQ ID NO: 1) peptide. At the end of the third week of culture, the generated T cell lines were evaluated by the ELISPOT assay for the capacity to release IFN-γ in response to peptide or autologous tumor stimulation, in the presence or in the absence of the anti- HLA-DR Ab L243. T cells were incubated with (1) medium, (2) LCL15392, (3) LCL15392 pulsed with 4 μg of PYYFAAELPP**R**NLPEP (SEQ ID NO: 1), (4) LCL15392 pulsed with 4 μg of PYYFAAELPP**R**NLPEP (SEQ ID NO:

1) and incubated with 10μg/ml of anti- HLA-DR L243 Ab, (5) LCL15392 pulsed with 2 μg of PYYFAAELPP**R**NLPEP (SEQ ID NO: 1), (6) LCL 15392 pulsed with 2 μg of PYYFAAELPP**R**NLPEP (SEQ ID NO: 1) and incubated with 10μg/ml of anti-HLA DR Ab L243, (7) LCL15392 pulsed with 4 μg of the wild type PYYFAAELPPGNLPEP (SEQ ID NO: 23) peptide, (8) autologous Me15392, (9) autologous Me15392 incubated with anti-HLA DR Ab L243.

Please delete the paragraphs on page 11, line 25 to page 12, line 18 and replace them with the following paragraphs:

HLA-DRB1*0102 and DRB1*1001 cloning. cDNA clones encoding the HLA-DRB1*0102 and DRB1*1001chains of pt15392 were obtained by RT-PCR starting from poly(A)$^+$ RNA prepared from 15392LCL. Amplification was performed using primers specific for conserved 5′ and 3′ regions of DRβ1 chains (forward 5′-CGCGGATCCAGCATGGTGTGTCTG-3 (SEQ ID NO: 3)′; reverse 5′-GGAATTCCTCAGCTCAGGAATCCTGTT-3′ (SEQ ID NO: 4)), and the PCR products were cloned into pcDNA3.1/V5-His TOPO vector (Invitrogen).

Construction and screening of the tumor-derived cDNA library. Poly(A)$^+$ RNA isolated from Me15392 cells using the FASTRACK kit (Invitrogen) was converted into cDNA with the Superscript Choise System (Life Technologies) using an oligo(dT) primer [5′-pGACTAGTTCTAGATCGCGAGCGGCCGCCC(T)$_{15}$-3′ (SEQ ID NO: 5)] containing a XbaI site (underlined). The cDNA was ligated to EcoRI-BstxI adaptors (Stratagene), digested with XbaI, and inserted into the XbaI and BstxI sites of the polylinker located at the 3′ end of

the invariant chain cDNA insert (Ii, amino acids 1-80), in the expression vector pEAK8.5/Ii, thus creating an Ii/tumor-derived fusion cDNA library. E. Coli DH5□ cells were transformed by electroporation with the recombinant plasmids and selected with ampicillin (0.1 g/liter). The library was divided into 1400 pools of about 100 cDNA recombinant clones each; pools were growth overnight in LB medium plus ampicillin (0.1 g/liter), and plasmid DNA was extracted using the QIAprep 96 plasmid kit (Qiagen).

Please delete the paragraphs on page 12, line 26 to page 13, line 16 and replace them with the following paragraphs:

Construction and sequencing of minigenes. cDNA #11 sequencing was performed with primers mapping into the regions of the pEAK8.5/Ii vector flanking the insert (forward 5'-ACCTCGATTAGTTCTCGAGCTT-3' (SEQ ID NO: 6), reverse 5'-ATTAGGACAAGGCTGGTGGGCACT-3' (SEQ ID NO: 7)). The non-conservative point mutation (g→a) was confirmed either by sequencing both DNA strands of amplified products from reverse-transcribed Me15392 poly(A)$^+$RNA (forward primer F2 5'-GTGCTCCTATCAGTGCTTAT-3' (SEQ ID NO: 8), reverse primer R2 5'-GCGTACGCACTGGGTTTT-3' (SEQ ID NO: 9)) or from Me15392 genomic DNA (forward primer 5'-CTGCACCCACACCGAACCAAGAGAGAA-3' (SEQ ID NO: 10), reverse primer 5'-CGCCTGGAAATAGATGTTGTATCCTTT-3' (SEQ ID NO: 11)). Mini-genes were prepared from cDNA #11 as PCR amplification products of different lengths. All the amplicons were obtained using the same sense primer F2 coupled with four different

antisense primers: EPR1 5'-CCGATTGTCACCCACAGTGAA-3' (SEQ ID NO: 12), EPR2 5'-GGGCAGGCTCAGGTA-3' (SEQ ID NO: 13), EPR3 5'-CTCGGGGGGAGTTCT-3' (SEQ ID NO: 14), and EPR2WT 5'-GGGCAGGCTCAGGTAGGTTTCCCG-3 (SEQ ID NO: 15)'. The latter was used for the preparation of the EP2wt minigene containing the wild type nucleotide (underlined in the EPR2WT primer). PCR products were cloned into pcDNA3.1/V5-His TOPO vector.

Please delete the paragraphs on page 13, line 21 to page 14, line 27 and replace them with the following paragraphs:

Northern Blot and RT-PCR analysis of R-PTPK expression. Poly(A)+ RNAs from Me15392, allogenic melanomas and PBL lines were isolated as described above. Total RNA was isolated by using RNAqueous™-4PCR kit (Ambion, Austin, TX, 78744, USA). For Northern blot experiments, 10 μg of each RNA sample was subjected to electrophoresis in a 1 % formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Inc. Piscataway, NJ 08855-1327, USA). The probes were labeled with [alpha-^{32}P]CTP by the random priming method (Amersham Biosciences), and pre-hybridization and hybridization were performed according to the Hybond-N+ paper guidelines. Membranes were washed four times with serially diluted solutions of SSC (from 0.03M to 0.0015M). Probes A and C were obtained by PCR amplification of Me15392 poly(A)$^+$ RNA with specific primers. Probe A, specific for the 5' region of the gene (bases 241-1110 of the gene), was

synthesized with primers forward F1 (5'-GGCGCTGCCTGCTTTTGT-3'; SEQ ID NO: 16) and reverse R1 (5'-GGAGGAGCAATGGGTCTT-3'; SEQ ID NO: 17). Probe C, specific for the region encoding the two intracellular phosphatase domains (bases 2925-4547 of the gene), was derived with primers forward F3 (5'-CTTGGGATGTAGCTAAAAAAGATCAAAATA-3' ; SEQ ID NO: 18) and reverse STOP (5'-CCAACTAAGATGATTCCAGGTACTCCAA-3' ; SEQ ID NO: 19). All the amplification products were sequenced before being used as probes. DNA clone #11 (bases 2084-2751 of the gene) was used as probe B. The RT-PCR analyses of PTPRK expression-profile in normal and tumors cell lines were performed with the forward primer F3 and with the reverse primer R□ (5'-CACCCTCTCTTTCAGCCAT-3' ; SEQ ID NO: 20) under the following conditions: 2' 94°C, 34 cycles consisting of 1' 94°C, 2' 54°C, 3' 72°C, and finally 10' 72°C. Conditions were set in order to obtain linear DNA amplification. The amplified DNAs were loaded on agarose gels, stained with ethidium bromide, and analyzed with a dedicated software (Image Master VDL-CS, Amersham Pharmacia Biosciences). Standard deviations were ≤ 5% on triplicate experiments. The level of expression of each sample was normalized for RNA integrity by taking into account the level of expression of the □-actine gene (reaction conditions: 4' 94°C, 21 cycles consisting of 1' 94°C, 2' 68°C, 2' 72°C, and finally 10' 72°C, corresponding to linear DNA amplification).

Please delete the paragraphs on page 15, lines 6-23 and replace them with the following paragraphs:

Epitope reconstitution assay. To analyze peptide recognition, 5×10^3 LCL15392 or LCL3700 cells were seeded in 96 microwells in 100 μl of RPMI 1640-10% PHS and then pulsed with different concentrations of the relevant peptide. Peptide loading was allowed to proceed for 2 h at 37°C before effector cells were added to give a final E:T ratio of 1:1. Supernatants were collected after 18 h and IFN-γ content was determined by ELISA (Mabtech AB, Stockholm, Sweden). Competition experiments were performed incubating 5×10^3 LCL15392 or 5×10^3 LCL3700 with various concentrations of the competitor peptides for 15 min before the addition of the PYYFAAELPPRNLPEP (SEQ ID NO: 1) peptide at 100nM. After 1 hr of additional incubation at 37°C, T cell clone was added at the final ratio of 1:1.

Generation of PTPRK specific T cells from pt15392 PBMCs. PBMCs of pt15392 obtained at 12 months after surgery, during the disease-free period of follow up, were stimulated in vitro with PYYFAAELPPRNLPEP (SEQ ID NO: 1) peptide as previously described (15). Briefly, PBMCs (2×10^6/well) were weekly stimulated with autologous peptide-pulsed monocytes. At the end of each stimulation, peptide-specific reactivity was monitored by Elispot assay.

Please delete the paragraph on page 21, line 10 to page 22, line 8 and replace it with the following paragraph:

These transfection experiments sharpened the potentially immunogenic amino acid region to a 26 amino acids-long peptide, shown in Fig. 4, that contained the mutation. To identify the TB515 epitope, several hexadecamer overlapping peptides were synthesized that spanned the identified 26 amino acid region. The peptides were evaluated in different doses for their ability to sensitize in vitro autologous LCL cells to recognition by the anti-melanoma TB515 clone. Among the hexadecamer peptides tested, PYYFAAELPP**R**NLPEP (SEQ ID NO: 1) ($PTPRK_{(667-682)}$), which extends from amino acid 667 to 682 of the protein and contains the mutation (Fig. 5, in bold), was the one giving rise to the strongest INF-γ release by TB515 clone. Indeed, titration experiments showed that this peptide could be detected by TB515 cells at a concentration as low as 10 nM, reaching its half-maximum effect at 100 nM (Fig. 5A). The substitution in the immunogenic peptide of the mutated arginine residue with the wild type glycine (Fig. 5A) completely abrogated stimulatory ability, thus definitely showing the relevance of the mutation for T cell mediated recognition. Further experiments with peptides progressively losing an amino acid residue at either the 5′ or the 3′ end (Fig. 5B and 5C) were aimed at identifying both the minimal epitope core and candidate anchor amino acids. In particular, the omission or the substitution with non relevant amino acids of Y_{669} (Fig. 5B) or L_{679} and N_{678} (Fig. 5C) strongly affected the capacity of the $PTPRK_{(667-682)}$ peptide to stimulate TB515 clone, thus suggesting a role for these amino

acids in position 3, 11 and 12 in the formation of the HLA-DR10/peptide/TCR complex. The wild type peptide PYYFAAELPP**G**NLPEP (SEQ ID NO: 23) and the PTPRK $_{(667-682)}$ with the Y_{669} substituted by a G were both able to bind the HLA-DR10, since in a competition assay they efficiently inhibited the recognition of the PTPRK$_{(667-682)}$ peptide by clone TB515 (Fig. 5D).

Please delete the paragraph on page 22, lines 17-27 and replace it with the following paragraph:

Pt15392 developed systemic immunity against the PTPRK derived, HLA-DR10 presented epitope. To establish whether pt15392 also developed a systemic, epitope-specific T cell immunity, PBMCs obtained 12 months after the surgical resection of the lymph node metastasis were cultured in vitro with PYYFAAELPP**R**NLPEP (SEQ ID NO: 1) peptide at 1 □M. After 3 weeks of in vitro stimulation with peptide-pulsed autologous monocytes, the cultured T cells showed a peptide-specific reactivity as detected by Elispot assay, indicating the presence in the peripheral blood of peptide-specific T cells (Fig. 7). These cells were also able to recognize the autologous tumor in an HLA-DR restricted fashion. Conversely, no specific reactivity was detectable in PBMCs obtained from DRB1*1001 positive healthy donors.